Bioaccumulation of ergovaline in bovine lateral saphenous veins in vitro

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Bioaccumulation of ergovaline in bovine lateral saphenous veins in vitro[1,2]

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ABSTRACT: Ergot alkaloids have been associated with vasoconstriction in grazing livestock affected by the fescue toxicosis syndrome. Previous in vitro investigations studying how ergot alkaloids caused vasoconstriction have shown that ergovaline has a distinct receptor affinity and sustained contractile response. A similar contractile response has not been noted for lysergic acid. The objectives of this study were to determine if repetitive in vitro exposure of bovine lateral saphenous vein to lysergic acid or ergovaline would result in an increasing contractile response and if a measurable bioaccumulation of the alkaloids in the vascular tissue occurs over time. Segments of vein were surgically biopsied from healthy, Angus × Brangus cross-bred, fescue-naïve yearling heifers (n = 16) or collected from healthy mixed breed and sex cattle immediately after slaughter (n = 12) at a local abattoir. Veins were trimmed of excess fat and connective tissue, sliced into cross-sections, and suspended in a myograph chamber containing 5 mL of oxygenated Krebs-Henseleit buffer (95% O_2/5% CO_2; pH = 7.4; 37°C). Contractile responses to repetitive additions of ergovaline (1×10^{-9} and 1×10^{-7} *M*) and lysergic acid (1×10^{-5} and 1×10^{-4} *M*) were evaluated using the biopsied veins. For the bioaccumulation experiments, veins collected at the abattoir underwent repetitive additions of 1×10^{-7} *M* ergovaline and 1×10^{-5} *M* lysergic acid and the segments were removed after every 2 additions and media rinses for alkaloid quantification via HPLC/mass spectrometry. Contractile data were normalized as a percentage of contractile response induced by a reference dose of norepinephrine (1×10^{-4} *M*). Repetitive additions of 1×10^{-9} *M* ergovaline and 1×10^{-5} and 1×10^{-4} *M* lysergic acid resulted in contractile response with a negative slope ($P < 0.02$). In contrast, repetitive addition of 1×10^{-7} *M* ergovaline resulted in a contractile response that increased with each addition ($P < 0.01$). Lysergic acid and ergovaline were detected at all 4 exposure levels (2× to 8×), but only the 1×10^{-7} *M* ergovaline treatment resulted in increased tissue content as the number of exposures increased ($P < 0.05$). These data indicate that ergovaline, but not lysergic acid, bioaccumulates with repetitive exposure in vitro. These results suggest that ergovaline may have a greater potential for inducing toxicosis in grazing animals than lysergic acid because of its potential to bioaccumulate at the cellular site of action.

Key words: bovine, ergovaline, lysergic acid, tall fescue, vasoconstriction

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INTRODUCTION

Consumption of toxic tall fescue (*Lolium arundinaceum*) alkaloids results in a reduction in peripheral blood flow (Rhodes et al., 1991; Aiken et al., 2007), morphological changes to vascular tissue (Williams et al., 1975; Strickland et al., 1996), and vasoconstriction (Oliver, 1997, 2005). Of the ergopeptine alkaloids produced by tall fescue endophyte, *Neotyphodium coenophialum*, ergovaline has been reported as the most abundant (Yates et al., 1985). This finding has fueled interest in ergovaline as a primary toxicant in the fescue toxicosis syndrome. Research using different vascular models and tissue culture systems has all reported a sustained contractile response to ergovaline (Dyer, 1993; Schön-

[1]Mention of trade name, proprietary product, or specified equipment does not constitute a guarantee or warranty by the USDA and does not imply approval to the exclusion of other products that may be suitable.

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ing et al., 2001; Klotz et al., 2007). These findings suggest that ergovaline has such a high binding affinity to a vascular receptor that it approaches irreversibility (Dyer, 1993). Thus, if the binding affinity of ergovaline is strong enough to permit a postabsorptive accumulation through a delayed clearance, then a gradual systemic build up in herbivores might occur because they are subject to more chronic exposure through grazing.

When evaluating the effects of selected combinations of tall fescue alkaloids on the contractile response of bovine lateral saphenous veins, Klotz et al. (2008) observed what appeared to be an additive effect of 1×10^{-7} *M* ergovaline, either in the presence of *N*-acetylloline or lysergic acid. However, the effect was not conclusive due to the presence of other alkaloids. From these observations, it was hypothesized that ergovaline, but not lysergic acid, accumulates in vascular tissue repeatedly exposed in vitro. Thus, the objectives of this study were to 1) determine if the contractile-response would gradually increase in response to a repetitive exposure of lysergic acid or ergovaline, and 2) determine if this repetitive exposure of ergovaline, lysergic acid, or both results in a measurable bioaccumulation in an in vitro bovine lateral saphenous vein bioassay.

MATERIALS AND METHODS

Methods used for the biopsies were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Animals and Tissues

Lateral saphenous vein (cranial branch) tissue used in this study was collected via biopsy (for the repetitive addition experiments) or from cattle at local abattoirs (for the bioaccumulation experiments) immediately after slaughter. Lateral saphenous veins used for the repetitive addition of lysergic acid and ergovaline experiments were biopsied from fescue-naïve Angus × Brangus crossbred heifers (n = 16; 305 ± 9 kg; obtained from the USDA-ARS, Dale Bumpers Small Farms Research Center, Booneville, AR) using methods reported by Klotz et al. (2008). Heifers were maintained in a drylot on a corn silage diet before biopsy. The biopsy consisted of placing the animal in a left lateral recumbency using a tilt table (Spring-O-Matic Inc., Marion, KS), and the biopsy site was clipped free of hair, cleaned (povidone-iodine soap solution), disinfected (70% ethyl alcohol), and locally anesthetized (lidocaine, 2% injectible; The Butler Co., Dublin, OH). A 10-cm incision was made in the tarsal region, slightly above and parallel to the target vein. After subcutaneous identification of the cranial branch of the lateral saphenous vein, ligatures were placed after the division of the lateral saphenous vein into cranial and caudal branches and before the cranial branch merged with a branch of the cranial tibial vein. The isolated venous tissue was excised and placed in a modified-Krebs Henseleit oxygenated buffer solution (95% O_2/5% CO_2; pH = 7.4; m*M* composition = D-glucose, 11.1; $MgSO_4$, 1.2; KH_2PO_4, 1.2; KCl, 4.7; NaCl, 118.1; $CaCl_2$, 3.4; and $NaHCO_3$, 24.9; Sigma Chemical Co., St. Louis, MO) for transport and kept on ice until processed (<3 h). Immediately after biopsy, heifers received penicillin (Procaine G, 6,600 U/kg of BW; Norbrook Inc., Kansas City, MO) and flunixin meglumine (Flunixiject, 1.1 mg/kg of BW; IVX Animal Health Inc., St. Joseph, MO) and were returned to the drylot for observation. The administration of flunixin meglumine continued for 2 d postbiopsy.

As a follow-up to the repetitive addition experiments, the ergovaline and lysergic acid bioaccumulation experiments were conducted separately using venous tissue collected from cattle of mixed breed and sex (n = 12; 419 ± 38 kg) at local abattoirs as described by Klotz et al. (2006) and Solomons et al. (1989). Other than the dissection procedure, these saphenous vein segments were obtained from the same region and transported in a modified Krebs-Henseleit buffer as described above.

Tissue processing was the same for all vein segments collected in this study and consisted of removal of excess fat and connective tissue. The cleaned segment was sliced into 2- to 3-mm cross-sections. Cross-sections were examined under a dissecting microscope (Stemi 2000-C, Carl Zeiss Inc., Oberkochen, Germany) at 12.5× magnification to measure dimensions for assurance of consistent segment size and to verify physical integrity of tissue. Cross-sections were suspended horizontally in a 5-mL tissue bath (DMT610M Multichamber myograph, Danish Myo Technologies, Atlanta, GA) containing continuously oxygenated modified-Krebs Henseleit buffer (95% O_2/5% CO_2; pH = 7.4; 37°C), with 3×10^{-5} *M* desipramine and 1×10^{-6} *M* propranolol (Sigma Chemical Co.) to inactivate catecholamine-neuronal uptake and β-adrenergic receptors, respectively. After equilibration to 1 g of tension (~1.5 h), tissues were exposed to the α-adrenergic agonist norepinephrine (1×10^{-4} *M*; Sigma Chemical Co.) to verify tissue viability and as a reference for normalization of the corresponding contractile responses.

Repetitive Addition Experiments

Cross-sections of lateral saphenous veins were run in duplicate from each animal (n = 6) for each alkaloid experiment. After recovery from the 1×10^{-4} *M* norepinephrine addition (45 to 60 min) and the reestablishment of the 1-g baseline tension, alkaloid additions occurred in 15-min intervals. Each 15-min interval consisted of a 9-min incubation period, followed by a washout period during which duplicate aliquots of buffer minus the alkaloid were incubated with the vein segment for two 2.5-min periods, followed by a final buffer replacement and 1-min incubation before the next addition. The repetitive alkaloid addition experiments were 8 consecutive additions every 15 min of 1 alkaloid at a fixed concentration. The alkaloid concentrations were based on results published by Klotz et al. (2008),

looking at contractile responses to combinations of alkaloids, or previously reported individual responses to lysergic acid (Klotz et al., 2006) or ergovaline (Klotz et al., 2007). The current experiments were conducted using biopsied fescue-naïve lateral saphenous vein cross-sections exposed to 1×10^{-5} and 1×10^{-4} *M* D-lysergic acid dihydrate (95%; Acros Organics, Geel, Belgium) and 1×10^{-9} and 1×10^{-7} *M* ergovaline tartrate (93%; supplied by F. T. Smith, Auburn University, AL). After the eighth addition of an alkaloid, the experiment was concluded with a final addition of 1×10^{-4} *M* norepinephrine to verify that the vein cross-sections were still viable at the conclusion of the experiment. The vein sections were removed from the myograph and frozen at −80°C until alkaloid extractions were conducted.

Bioaccumulation Experiments

The alkaloid bioaccumulation experiments were equilibrated, exposed to norepinephrine, and followed the same incubation and buffer rinse time intervals as described for the repetitive addition experiments. The treatment additions consisted of consecutive additions every 15 min of 1 alkaloid at a fixed concentration, with 2 venous cross-sections removed from the myograph every 2 additions (30-min intervals). These experiments were conducted using abattoir-obtained lateral saphenous vein cross-sections exposed to 1×10^{-7} ergovaline tartrate or 1×10^{-5} *M* D-lysergic acid dihydrate. After every 2 alkaloid additions, 2 vein sections were removed from the myograph resulting in 2×, 4×, 6×, and 8× exposures to each alkaloid. Upon removal from the myograph, vein sections were frozen at −80°C until alkaloid extractions were conducted. The nature of these experiments prevented the final addition of 1×10^{-4} *M* norepinephrine to verify tissue viability.

Extraction and Liquid Chromatography/Mass Spectrometry Quantification of Lysergic Acid and Ergovaline

The lateral saphenous vein tissue samples were freeze-dried (Botanique Preservation Equipment Inc., Peoria, AZ), weighed, and extracted in 1 mL of HPLC-grade methanol in an incubator shaker (Innova 4300, New Brunswick Scientific Co. Inc., New Brunswick, NJ) shaking at 350 rpm at 30°C for 2 h. The vein-containing tubes were centrifuged (2,500 × *g* at 30°C for 10 min), and the methanol supernatant was transferred to an amber autosampler vial. The methanol was removed from the sample by drying under a N_2 stream, and the alkaloid-containing residue was redissolved in 200-uL of initial mobile phase solution (95% H_2O, 5% acetonitrile, and 0.1% formic acid, vol/vol) for analysis.

The quantitative determination of ergovaline and lysergic acid was accomplished using reverse phase HPLC and detection with a Varian 1200L triple quadrupole mass spectrometer (**MS**; Walnut Creek, CA). The HPLC system consisted of a ProStar 430 autosampler, 2 ProStar 210 solvent delivery modules and a 150 × 2.1 mm Gemini 5 μm C18 column (Phenomenex, Torrance, CA). The eluent from the HPLC column was directly coupled to the Varian 1200L MS using an electrospray ionization source. Fifty-microliter (partial loopfill) aliquots of sample extracts or standard solutions were injected onto the C18 column. The column was equilibrated with 5% B and initially held for 1 min, followed by a linear increase to 30% B over 28 min to chromatographically resolve the ergopeptides. The gradient was held at 95% B for the next 5 min to wash the column and finally re-equilibrated at 5% B for 9 min to re-equilibrate the liquid chromatography column for the next analysis. The flow was 0.2 mL/min. The eluent from the column was directed to the electrospray ionization source of the MS under conditions used to generate protonated ions. Electrospray ionization settings included a needle potential and current of 5,000 V and 18 μA, respectively. Ultra-high-purity N was used as drying and nebulizing gas set at 131 kPa/200°C and 310 kPa/50°C, respectively. The capillary potential was 60 V, and the shield was 600 V. Mass analysis was performed using single ion monitoring in which the current for singly protonated molecular ions was detected with an electron multiplier setting of −1,800 V.

The singly protonated molecular ions of lysergic acid (*m/z* 269), ergovaline, (*m/z* 534), and methysergide (*m/z* 354; internal standard; >99%; Sigma Chemical Co.) were used for identification and quantification of the alkaloids during HPLC-MS analysis. Aliquots of matrix solution containing known concentrations of lysergic acid, ergovaline, and methysergide were analyzed as described previously. Standard concentration ranges for lysergic acid and ergovaline ranged from 0.25 to 30 pmol injected on column. Each standard solution also contained 10 μ*M* methysergide. Preceding and concluding the analysis of a sample set was the injection of a quality control matrix solution that was created from a large quantity of lateral saphenous vein simultaneously exposed to 2×10^{-5} *M* lysergic acid and ergovaline in a modified Krebs-Henseleit buffer solution for 15 min. The vein segments were then frozen at −20°C, freeze-dried, and stored until needed for analysis.

Data Collection and Analysis

For both the repetitive addition and bioaccumulation experiments, isometric contractions were recorded as grams of tension in response to exposure to norepinephrine and the subsequent addition of ergovaline or lysergic acid. Data were digitized and recorded using a Powerlab/8sp and Chart software (version 5.5, ADInstruments, Colorado Springs, CO). The contractile responses were recorded as the greatest grams of tension detected within the 9-min incubation period. All maximal values measured were corrected by the baseline measured during the interval just preceding the addition of the norepinephrine (1×10^{-4} *M*) reference treatment, thus generating cumulative contractile responses.

To compensate for variation of tissue responsiveness due to differences in tissue size or individual animal variation, values were normalized as a percentage of the maximal contraction produced by norepinephrine. Contractile response data are presented as percentage means ± SE of the maximal contraction induced by norepinephrine and plotted to illustrate the response of the bovine lateral saphenous vein.

When determining ergovaline or lysergic acid concentrations from the end-point samples of the repetitive addition experiments or the increasing exposure samples of the bioaccumulation experiments, calibration curves were generated by plotting the HPLC peak area ratio of the analyte (lysergic acid or ergovaline) to the internal standard (methysergide) vs. the injected analyte mass. The peak area for total ergovaline was calculated by adding the peak areas for ergovaline and ergovalinine. Best fit of a least squares linear regression line on each calibration curve was used to determine the linear dynamic range for each analyte. The calculated mass of a sample analyte was adjusted by the beginning mass of vein sample that resulted in alkaloid quantification to be expressed as picograms or nanograms of alkaloid per milligram of vein DM. The quantitative determination of ergovaline and lysergic acid from quality control vein sections permitted within and between run variation to be evaluated. A typical sample set run consisted of 6 standards, 8 unknown vein samples, and 2 quality control injections at 43 min per injection or a total of 11.4 h. Intra- and interassay CV for lysergic acid and ergovaline were 3.7 and 1.7, and 9.8 and 14.2%, respectively.

Statistical Analysis

Student's *t*-tests were used to ascertain whether slopes of the contractile responses of lateral saphenous veins to repetitive additions of lysergic acid or ergovaline treatments differed from zero for both experiments. The quantities of lysergic acid and ergovaline recovered from the saphenous veins used in the bioaccumulation experiments were separately evaluated to determine if an actual bioaccumulation could be detected by the number of exposures. Data for each alkaloid were analyzed using a completely randomized design ($Y_{ij} = \mu + T_i + e_{ij}$, where Y_{ij} = the dependent variable, μ = the overall mean, T_i = the effect of treatment i, and e_{ij} = residual error). Experimental units were the incubation chambers of the myograph, and the number of alkaloid additions added to the chamber was the treatment. Data reported are means of each treatment incubated and quantified in duplicate from 6 different saphenous vein preparations (animals; except for 2 × ergovaline, which only had 5 animals, due to a problem with the internal standard). The model utilized the mixed model procedure (SAS Inst. Inc., Cary, NC). ANOVA was conducted, and pairwise comparisons of least square means (±SEM) were performed (SAS Inst. Inc.) if the probability of a greater *F*-statistic was significant. Effects and comparisons were considered different at $P \leq 0.05$.

RESULTS

Repetitive Additions of Lysergic Acid and Ergovaline

Exposure of biopsied tall fescue-naïve bovine lateral saphenous veins to repetitive additions of 1×10^{-5} and 1×10^{-4} *M* lysergic acid and 1×10^{-7} and 1×10^{-9} *M* ergovaline resulted in distinct contractile responses between concentrations and alkaloid type evident in the traces presented in Figure 1. Specifically, 1×10^{-5} *M* lysergic acid and 1×10^{-9} *M* ergovaline treatments did not show any response compared with the sustained contractile response that was visible for 1×10^{-7} *M* ergovaline treatment. Conversely, the trace in Figure 1 that represents the repetitive additions of 1×10^{-4} *M* lysergic acid demonstrates a declining contractile response with each addition. The final addition of 1×10^{-4} *M* norepinephrine confirmed that all vein cross-sections were still responsive at the end of the experiments (Figure 1), thereby confirming tissue viability throughout the experiments.

Normalized data for the repetitive additions of lysergic acid were plotted as contractile response as a percentage of the corresponding norepinephrine maximum against the number of additions and best of fit polynomial curves were calculated for both concentrations (Figure 2A). For both 1×10^{-5} and 1×10^{-4} *M* lysergic acid, the response was quadratic and the $\times^2$ coefficient was positive. The slopes for percent contractile response over number of additions were negative for both concentrations (Figure 2A) and different from zero ($P < 0.02$). The mean contractile response to the initial (1×) addition of 1×10^{-4} *M* lysergic acid was 25.3 ± 5.1% of the norepinephrine maximum, whereas the contractile response to the final (8×) addition was 15.6 ± 3.0% of the norepinephrine maximum. This trend was similar for the repetitive additions of 1×10^{-5} *M* lysergic acid, but in this instance the tension relaxed below the baseline resulting in a negative contractile response (1× = 4.4 ± 1.4% and 8× = −1.7 ± 0.5% of the norepinephrine maximum). After the concluding addition of norepinephrine, duplicate cross-sections from 3 of the biopsied animals were retained for liquid chromatography/MS analysis of the presence of lysergic acid. A chromatogram of the 1×10^{-4} and 1×10^{-5} *M* lysergic acid overlaid peaks demonstrates a greater recovery of the 1×10^{-4} *M* exposed cross-sections compared with the 1×10^{-5} *M* (Figure 2B). Correspondingly, the 8× quantities of lysergic acid recovered from 1×10^{-5} and 1×10^{-4} *M* were 650.1 ± 114.5 and 4,515.4 ± 633.2 pg/mg of vein DM, respectively.

Normalized data for the repetitive additions of ergovaline were plotted as contractile response as a percentage of the corresponding norepinephrine maximum against the number of additions, and best of fit polynomial

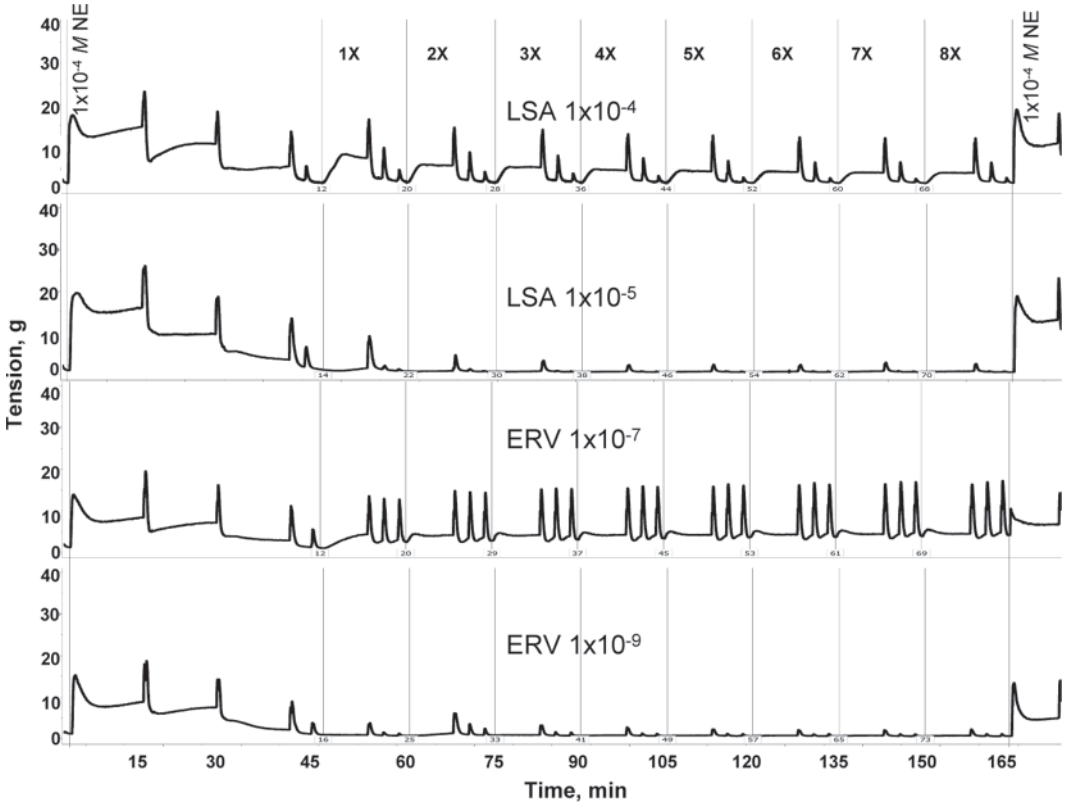

Figure 1. Example of typical contractile responses of isolated fescue-naïve bovine lateral saphenous vein sections to repetitive additions of 1×10^{-4} and 1×10^{-5} *M* lysergic acid (LSA) and 1×10^{-7} and 1×10^{-9} *M* ergovaline (ERV). These are complete data recordings on the myograph that include the initial 1×10^{-4} *M* norepinephrine (NE) addition, the alkaloid additions that are demarcated by dashed lines with the addition number at the top of the figure (1X to 8X), and the concluding addition of NE. The spikes that precede an addition are artifacts generated from buffer replacement and were not included in the data collection and analysis.

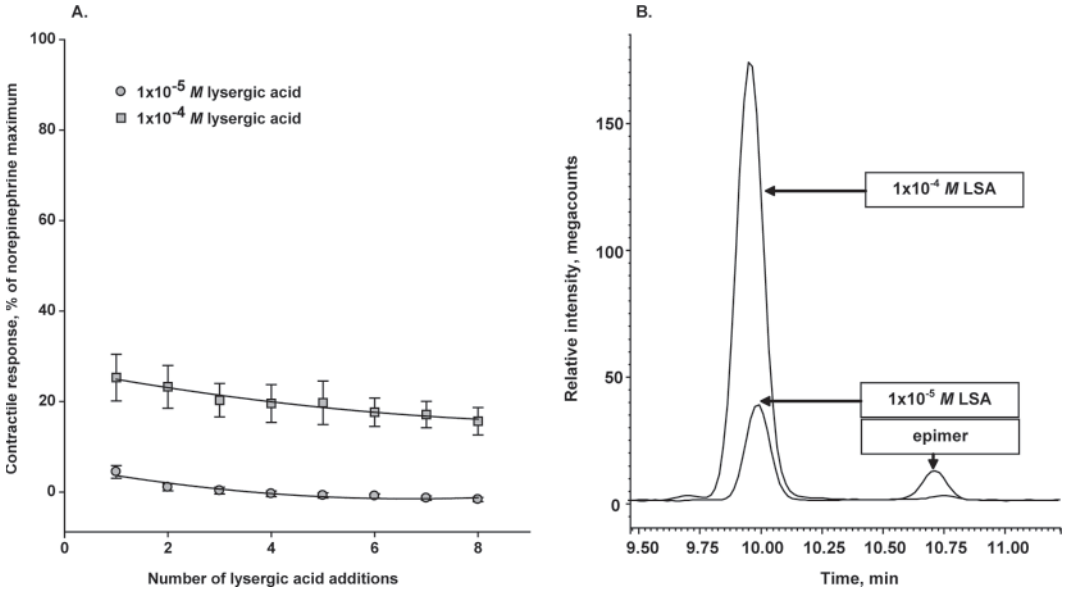

Figure 2. A) Mean contractile responses of bovine lateral saphenous veins from tall fescue-naïve heifers to repetitive additions of 1×10^{-5} *M* (gray circles; n = 6) and 1×10^{-4} *M* (gray squares; n = 6) lysergic acid (LSA). The best of fit polynomial lines demonstrate the effect of addition number on contractile response. Line equations are $y = 0.15x^2 - 2.12x + 5.61$; $r^2 = 0.91$ for 1×10^{-5} *M* LSA and $y = 0.1x^2 - 2.16x + 26.99$; $r^2 = 0.95$ for 1×10^{-4} *M* LSA. B) Total ion chromatogram for LSA extracted from bovine lateral saphenous veins from tall fescue-naïve heifers exposed to repetitive additions of 1×10^{-5} or 1×10^{-4} *M* LSA (650.1 ± 114.5 and 4,515.4 ± 633.2 pg/mg of vein DM, respectively; n = 3). The singly protonated molecular ion for LSA is plotted ($m/z = 269$), as analyzed using HPLC coupled to mass spectrometry.

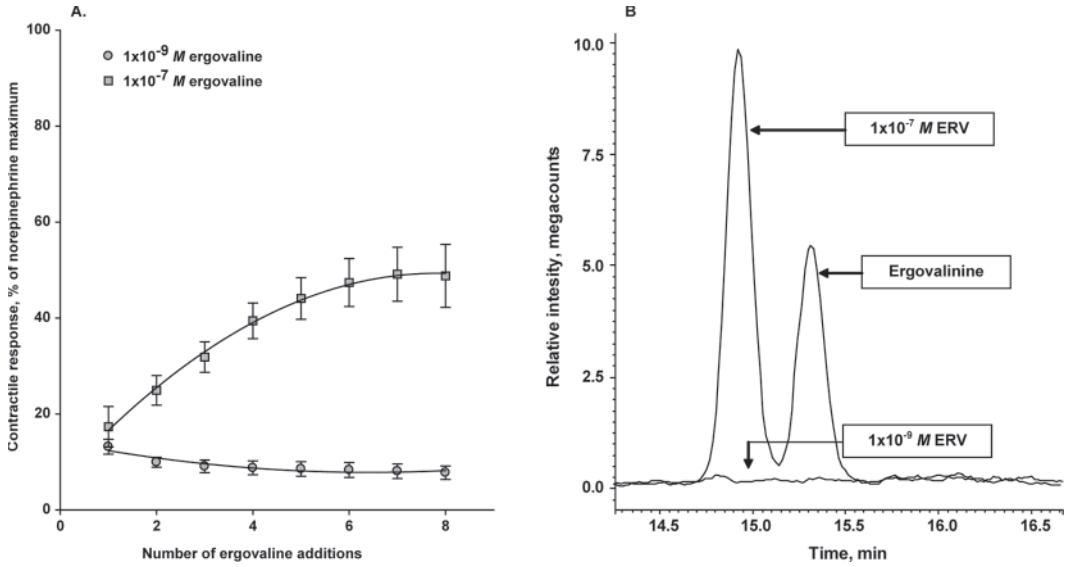

Figure 3. A) Mean contractile responses of bovine lateral saphenous veins from tall fescue-naïve heifers to repetitive additions of 1×10^{-9} (gray circles; n = 6) and 1×10^{-7} (gray squares; n = 6) *M* ergovaline (ERV). The best of fit polynomial lines demonstrate the effect of addition number on contractile response. Line equations are $y = 0.15x^2 - 1.98x + 14.18$; $r^2 = 0.88$ for 1×10^{-9} *M* ERV and $y = -0.7x^2 + 11.01x + 6.28$; $r^2 = 0.99$ for 1×10^{-7} *M* ERV. B) Total ion chromatogram for ERV extracted from bovine lateral saphenous veins from tall fescue-naïve heifers exposed to repetitive additions of 1×10^{-9} or 1×10^{-7} *M* ERV (not detected and 531.9 ± 71.6 pg/mg of vein DM, respectively; n = 3). The singly protonated molecular ion for ERV and the epimer, ergovalinine, are plotted ($m/z = 534$), as analyzed using HPLC coupled to mass spectrometry.

curves were calculated for both concentrations (Figure 3A). The contractile responses of 1×10^{-9} and 1×10^{-7} *M* ergovaline were quadratic, but the $\times^2$ coefficient was positive for 1×10^{-9} and negative for 1×10^{-7} *M* (a result of the asymptote apparent at the 7× and 8× additions). The curve for the repetitive additions of 1×10^{-9} *M* ergovaline (Figure 3A) resemble those for the repetitive additions of lysergic acid (Figure 2A) with a negative slope (1× = 13.7 ± 1.5% and 8× = 7.8 ± 1.4% of the norepinephrine maximum; significantly different from zero, $P = 0.01$), but the contractile response to repetitive additions of 1×10^{-7} *M* ergovaline had a positive slope (Figure 3A; 1× = 17.4 ± 4.2% and 8× = 48.8 ± 6.6% of the norepinephrine maximum significantly different from zero, $P < 0.001$). The extraction and quantification of ergovaline from 8×-exposed duplicate cross-sections from 3 biopsied animals resulted in only the detection of 1×10^{-7} *M* ergovaline (Figure 3B; 531.9 ± 71.6 pg/mg of vein DM). There was no ergovaline detected in the extracts from the 1×10^{-9} *M* ergovaline samples (Figure 3B).

Bioaccumulation of Ergovaline

Treatment of bovine saphenous veins collected from abattoir animals with an increasing number of exposures to 1×10^{-5} *M* lysergic acid and 1×10^{-7} *M* ergovaline resulted in different contractile responses (Figure 4). These differing responses resembled those observed with the biopsied fescue-naïve venous segments (Figures 2A and 3A). The contractile response to increasing number of exposures to 1×10^{-5} *M* lysergic acid resulted in a recurring to a decreasing response with each subsequent addition (Figure 4; 1× = 3.1 ± 0.5% to 8× = −0.9 ± 0.9% of the norepinephrine maximum). Whereas, the contractile response to increasing number of exposures to 1×10^{-7} *M* ergovaline resulted in a gradual, but steady, increase in response to each addition (Figure 4; 1× = 8.8 ± 2.7% to 8× = 30.5 ± 9.3% of the norepinephrine maximum).

Selected venous cross-sections were removed from the myograph every 2 additions, and the relationship between removal and contractile response to repeated additions of 1×10^{-5} *M* lysergic acid is illustrated in Figure 5A. The total ion chromatogram (Figure 5B) that corresponds with the contractile response demonstrates a typical recovery for the 2×, 4×, 6×, and 8× lysergic acid (1×10^{-5} *M*) exposures. There was never a consistent order when looking at peak height. Conversely, the trace presented in Figure 6A closely resembles the contractile response presented in Figure 1 for repetitive additions of 1×10^{-7} *M* ergovaline in biopsied fescue-naïve lateral saphenous veins. The trace in Figure 6A illustrates the removal points of the veins from the myograph, and a steadily increasing response is more apparent. This is reflected in the quantity of ergovaline recovered and resulted in a consistently increasing peak height that correlated with increasing number of additions (Figure 6B). The second smaller peak in the chromatogram is the isomer of ergovaline, ergovalinine.

The chromatogram in Figure 5B demonstrates that the number of 1×10^{-5} *M* lysergic acid exposures did not translate to a difference in the lysergic acid in the extract. The mean values presented in Table 1 are total alkaloid concentrations representing the alkaloids and their corresponding epimer. There was no difference between number of additions of 1×10^{-5} *M* lysergic

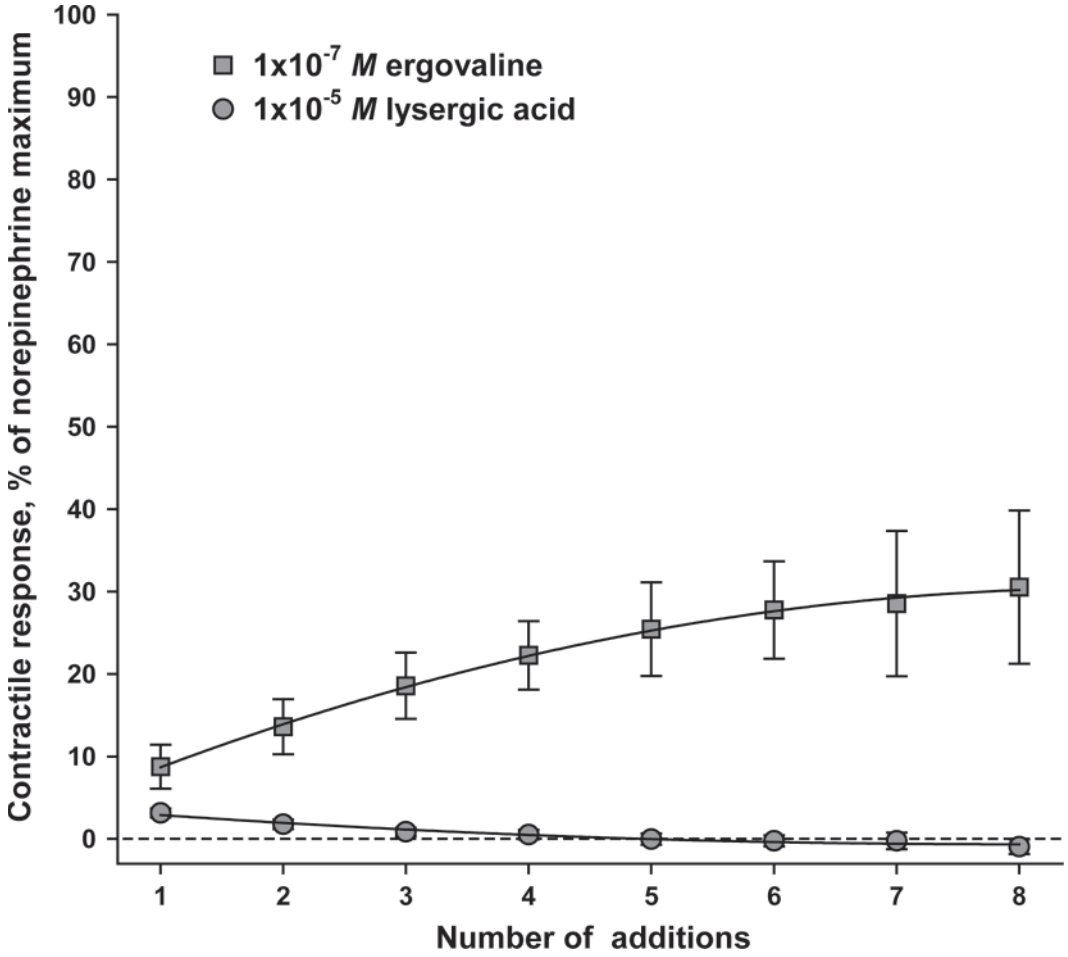

Figure 4. Mean contractile responses of bovine lateral saphenous veins from cattle of mixed breed and sex to repetitive additions of 1×10^{-5} *M* lysergic acid (gray circles; n = 6) and 1×10^{-7} *M* ergovaline (gray squares; n = 6). Line equations are $y = 0.08x^2 - 1.18x + 4.01$; $r^2 = 0.97$ for 1×10^{-5} *M* lysergic acid and $y = -0.36x^2 + 6.3x + 2.75$; $r^2 = 0.99$ for 1×10^{-7} *M* ergovaline.

acid (e.g., 2× = 9.1 ± 0.9 and 8× = 9.2 ± 0.9 ng/mg of vein DM). Conversely, the chromatogram in Figure 6B clearly illustrates an increasing concentration of ergovaline recovered in the extracts as the number of exposures increases. This is again reflected in the mean values presented in Table 1 for repetitive additions of 1×10^{-7} *M* ergovaline (e.g., 2× = 309.9 ± 32.5 pg/mg of vein and 8× = 523.6 ± 52.3 pg/mg of vein), with the 6× and 8× values being greater than the 2× and 4× values ($P < 0.05$).

DISCUSSION

Repetitive Exposure of Alkaloids

The treatment concentrations utilized in these experiments were selected based on the vascular sensitivity (the concentration of an agonist at which onset of contraction is observed) of ergovaline (1×10^{-8} *M*; Klotz et al., 2007) and lysergic acid (1×10^{-5} *M*; Klotz et al., 2006). Thus, the repetitive additions of 1×10^{-9} and 1×10^{-7} *M* ergovaline were testing the lesser and greater concentrations on either side of this previously reported sensitivity, and the 2 lysergic acid concentrations were chosen because they were the only 2 that resulted in a contractile response in previous work (Klotz et al., 2006). Also, the examination of 1×10^{-5} and 1×10^{-7} *M* lysergic acid and ergovaline (respectively) clarified results reported by Klotz et al. (2008) when studying the effects of alkaloids in combination. Specifically, there appeared to be an additive effect when 1×10^{-7} *M* ergovaline was included as a treatment, but due to the confounding presence of other alkaloids this was only speculative. In the current study, the repetitive additions of 1×10^{-7} *M* ergovaline resulted in an increasing contractile response and the additions of 1×10^{-5} *M* lysergic acid resulted in a tachyphylaxsis-like or decreasing contractile response. This reproduction of the observed contractile responses reported by Klotz et al. (2008) in absence of additional alkaloids confirmed that there was some type of additive effect or bioaccumulation occurring when venous cross-sections were repeatedly exposed to ergovaline, but not lysergic acid within this in vitro bioassay. The apparent lack of response to the repetitive addition of 1×10^{-9} *M* ergovaline and the elevated, but parallel (to 1×10^{-5} *M*) response to 1×10^{-4} *M* lysergic acid prompted the preliminary evalua-

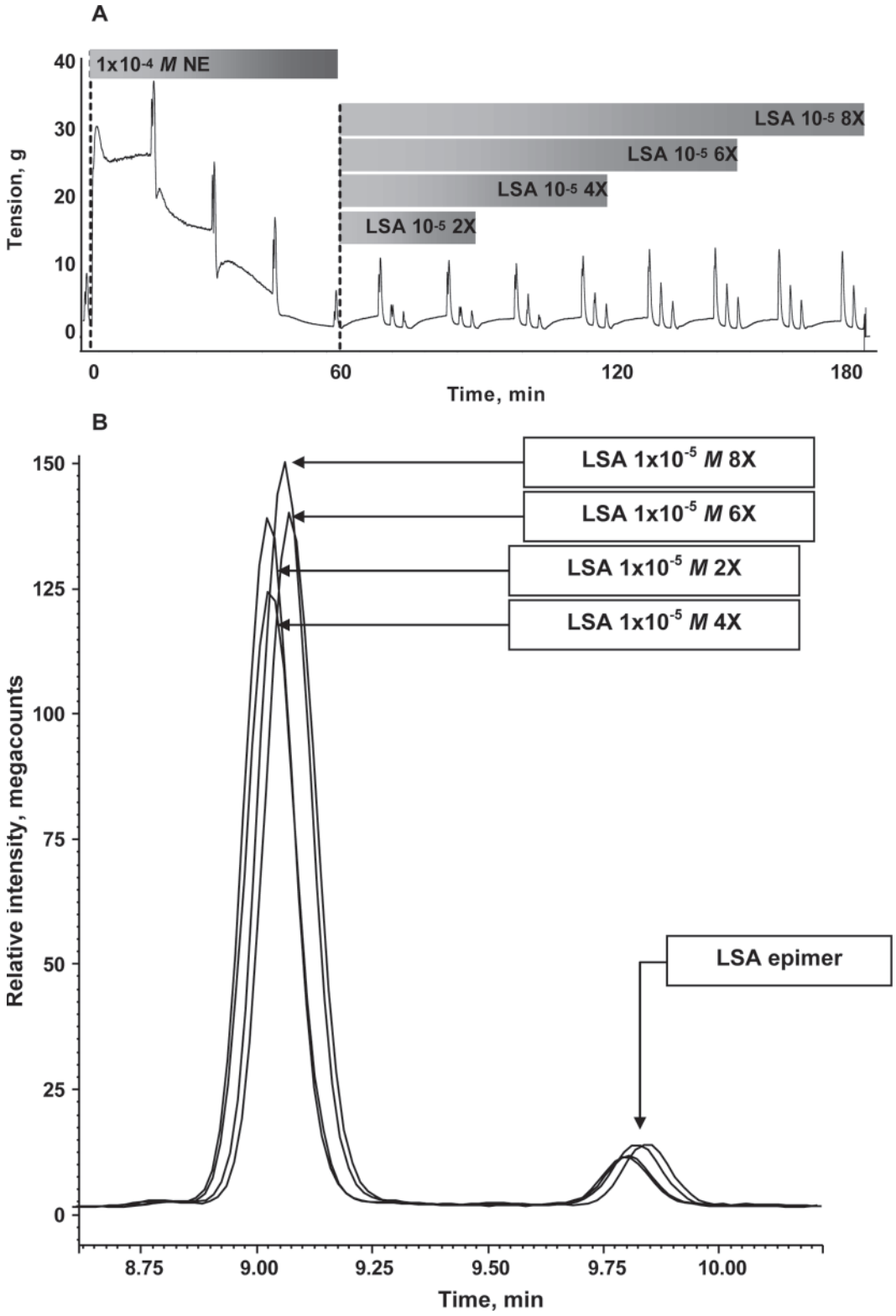

Figure 5. A) Typical contractile response to repeated additions (illustrated as 2X, 4X, 6X, and 8X) of 1×10^{-5} *M* lysergic acid (LSA). The initial portion of the trace is the contractile-response to 1×10^{-4} *M* norepinephrine (NE). B) Total ion chromatogram for lysergic acid, as extracted from vein sections exposed an increasing number of times to 1×10^{-5} *M* lysergic acid. The singly protonated molecular ion for lysergic acid ($m/z = 269$) is plotted.

tion of end-point quantification of the bioaccumulation of these alkaloids using HPLC/MS.

The results of the end-point (exposed 8×) extractions of saphenous vein cross-sections repeatedly exposed to 1×10^{-5} and 1×10^{-4} *M* lysergic acid or 1×10^{-9} and 1×10^{-7} *M* ergovaline confirmed not only the ability to extract and quantitate both alkaloids from bovine venous tissue, but that there were differences related

to exposure concentrations. The repetitive addition of 1×10^{-9} *M* ergovaline did not result in any cumulative contractile response or a measurable quantity that was distinguishable from the baseline noise. Conversely, the 1×10^{-7} *M* additions resulted in a very distinct signal that lead to this concentration being selected for the bioaccumulation portion of the study. Correspondingly, measurable quantities of lysergic acid were extracted from both concentrations of lysergic acid-exposed veins and produced significant peaks. The 1×10^{-5} *M* lysergic acid treatment was selected for the bioaccumulation portion of the study because it is the concentration when a contractile response is first observed and 1×10^{-4} *M* lysergic acid is considered a supraphysiological

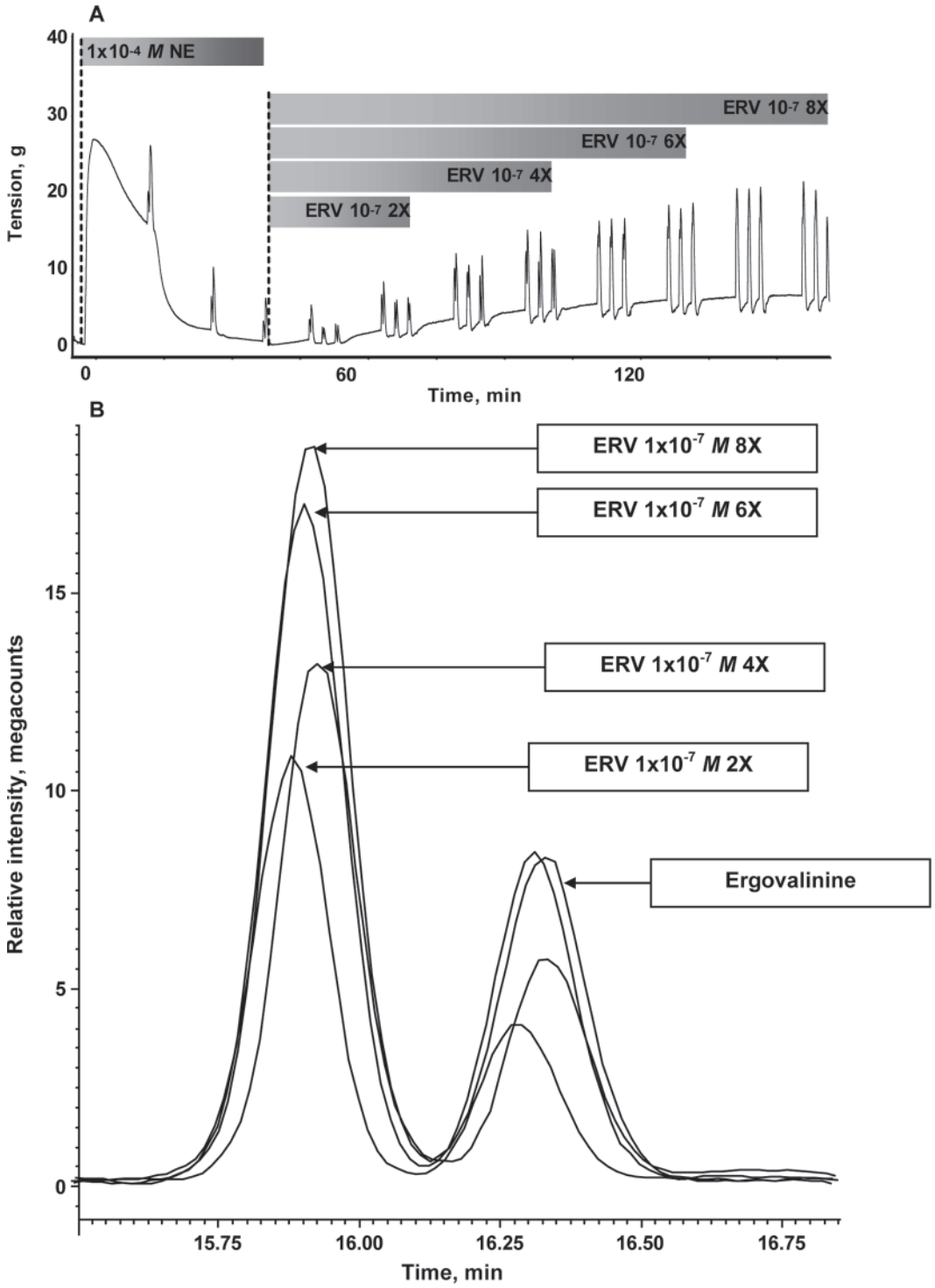

Figure 6. A) Typical contractile response to repeated additions (illustrated as 2X, 4X, 6X, and 8X) of 1×10^{-7} *M* ergovaline (ERV). The initial portion of the trace is the contractile-response to 1×10^{-4} *M* norepinephrine (NE). B) Total ion chromatogram for ergovaline, as extracted from vein sections exposed an increasing number of times to 1×10^{-7} *M* ergovaline. The singly protonated molecular ion for ergovaline ($m/z = 534$) and its epimer, ergovalinine, are plotted.

Table 1. Means and SE of ergovaline (pg/mg of vein DM) or lysergic acid (ng/mg of vein DM) extracted and quantified from sections of bovine lateral saphenous vein exposed to an increasing number of additions in vitro[1]

	Alkaloid exposure	
Number of exposures	Ergovaline, 1×10^{-7} *M*	Lysergic acid, 1×10^{-5} *M*
2×	309.9[b] ± 41.9	9.1[a] ± 0.9
4×	362.9[b] ± 38.3	9.4[a] ± 0.9
6×	479.6[a] ± 38.3	9.0[a] ± 0.9
8×	523.6[a] ± 38.3	9.2[a] ± 0.9

[a,b]Means without a common superscript within a column are different ($P < 0.05$).

[1]All means are n = 6, except for the 2× ergovaline, which was n = 5. Means presented are total concentrations, meaning that the peak areas of the alkaloid and the corresponding epimer were combined.

concentration (Klotz et al., 2006). Thus, concentrations were evaluated within the range that elicited responses in the bovine lateral saphenous vein bioassay.

Does Ergovaline Bioaccumulate In Vitro?

Klotz et al. (2007) reported that the contractile response of cross-sections of bovine lateral saphenous veins to a single addition of 1×10^{-4} *M* ergovaline lasted over 90 min without returning to baseline tension despite routine buffer changes. Dyer (1993) reported that the contractile response of bovine uterine arteries to 1×10^{-8} *M* ergovaline lasted longer than 3 h without returning to baseline. Also, Schöning et al. (2001) reported that a contractile response of rat caudal arteries to 3×10^{-8} *M* ergovaline was diminished only slightly after a 60-min washout period. The reoccurrence of this phenomenon across different bioassays, different vessel types, and different species suggests that ergovaline has a very strong receptor affinity and dissociates very slowly. These reports combined with findings discussed above in this study, that repeated exposures of 1×10^{-7}, but not 1×10^{-9} *M* ergovaline led to an increasing contractile response and quantifiable extraction of ergovaline substantiate the idea that ergovaline could be accumulating in the blood vessels in vitro. In contrast, repetitive additions of only lysergic acid (in the current experiment) or in combination with *N*-acetylloline (Klotz et al., 2008) did not result in an increasing contractile response.

The corresponding hypothesis was that the quantitative determination of lysergic acid and ergovaline from the increasingly exposed saphenous vein sections would not differ for those exposed to lysergic acid, but would differ for those exposed to ergovaline. The quantitative data presented in the bioaccumulation experiment confirm this hypothesis. Specifically, lysergic acid was recovered in the extracts of saphenous veins repeatedly exposed on the myograph, but the number of exposures did not affect the measured concentrations extracted from those vein cross-sections. The reason it appeared that more lysergic was extracted from these 8× veins than the biopsied end-point veins was that the end-point veins were exposed to a concluding addition of norepinephrine that resulted in what amounted to an additional buffer rinse, whereas the 8× veins were removed from the myograph after the third rinse after the eighth addition.

Contrary to lysergic acid, the number of exposures of ergovaline resulted in a significant increase in concentrations measured in the corresponding extracts. Hill et al. (2001) reported absorption (although limited in relation to the less complex ergoline alkaloids) of ergopeptine alkaloids (e.g., ergovaline) across ruminant foregut tissues in vitro. Bioaccumulation of a toxicant may occur in an animal when the rate of absorption exceeds its clearance. Clearance may be hindered by sequestration in or binding of toxicants to tissues in vivo. Thus, if the binding affinity of ergovaline is strong enough to permit a postabsorptive accumulation through slowing of the clearance, then a gradual systemic buildup of select ergot alkaloids in grazing livestock subject to chronic exposure through grazing might occur. Realini et al. (2005) reported that cattle grazing wild-type endophyte-infected tall fescue had greater subcutaneous fat concentrations of ergot alkaloids than cattle grazing AR542 infected tall fescue. This report of alkaloid accumulation combined with reports of retained or metabolized [= intake – (fecal concentration + urine concentration)] ergovaline in geldings (Schultz et al., 2006), lambs (De Lorme et al., 2007), and steers (Merrill et al., 2007) indicate that the potential exists for bioaccumulation of ergovaline in vivo.

In conclusion, repetitive additions of lysergic acid did not result in increasing contractile response, and the quantity extracted from increasingly exposed venous cross-sections did not differ. Conversely, repetitive additions of 1×10^{-7} *M* ergovaline caused an increasing contractile response and resulted in a significant increase in ergovaline extracted from increasingly exposed saphenous veins. This is evidence that there is a bioaccumulative effect of repeated ergovaline exposures, but not lysergic acid, on saphenous veins exposed in vitro. Further work is necessary to determine if this phenomenon also occurs in the grazing animal, which is continuously exposed to varying concentrations and combinations of ergot alkaloids.

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